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Proteomic Analysis of the Balance between Survival and Cell Death Responses in Cisplatin-Mediated Ototoxicity

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Abstract

Cisplatin, a widely used anticancer drug, preferentially damages outer hair cells (OHCs) of the inner ear. In this study, an antibody microarray was used to identify early changes in protein expression in the rat cochlea induced by cisplatin. Only small changes in hearing thresholds (4–34 dB elevation) were detected two days after cisplatin treatment (12 mg/kg). OHC function, measured by otoacoustic emissions, was slightly depressed (10 dB), and little or no receptor cell loss was observed. However, cisplatin induced large changes in the expression of 19 proteins involved in apoptosis, cell survival, or progression through the cell cycle. Fifteen of the proteins are novel to the study of the inner ear. Immunoblotting confirmed increases in the levels of the pro-survival activating transcription factor 2 (ATF2), of pro-apoptotic serine-threonine protein kinase, receptor interacting protein, and a 70/75 kDa nitrotyrosine bearing doublet of unknown function. Anti-nitrotyrosine antibodies localized these oxidatively damaged proteins to the stereocilia of OHCs, the Golgi-centrosome region of Hensen's cells, nuclei of outer pillar cells, and tunnel crossing fibers innervating OHCs. The results of this proteomic analysis reflect the commencement of ototoxic and cell survival responses before the observation of a significant functional or anatomical loss.

Keywords

antibody microarray; cisplatin; cochlea; oxidative protein damage; protein tyrosine nitration

Introduction

The platinum atom of cisplatin forms covalent bonds with DNA at the N7 positions of purine bases to form intrastrand and interstrand cross-links. Platinated DNA immunoreactivity has been localized to the nuclei of outer hair cells and cells in the stria vascularis and spiral ligament.¹ Cytotoxic effects of cisplatin have been suggested to occur primarily through apoptosis at higher doses and through DNA damage at lower doses.² However, augmented pro-apoptotic signaling has been demonstrated in the cochlea within 48 h of treatment with relatively low doses of cisplatin.³ Cisplatin targets three areas in the cochlea: the hair cells in the basal turn

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of organ of Corti,⁴ the lateral wall tissues,^{5,6} and the spiral ganglion cells.¹ Cisplatin-induced generation of reactive oxygen species, including the superoxide anion⁷ is one of the important factors leading to cell death. Alternate death pathways implicate transcription factors, like NF- κ B and high mobility group protein.⁸

Genomic studies, including DNA arrays, have also been used to investigate cochlear mRNA expression in cisplatin-induced hearing loss. TNF- α was immunolocalized to the spiral ligament, spiral limbus, and the organ of Corti, while mRNA expression increased in HEI-OCI cells treated with cisplatin.⁹ Cisplatin treatment induced kidney injury molecule-1 mRNA expression and increased NOX-3 mRNA in rat cochlea and hair cell lines derived from mouse.¹⁰ Immunocytochemistry also revealed very strong expression of NF- κ B p65 in cells of organ of Corti, spiral ligament, and stria vascularis where cisplatin-induced TUNEL-positive staining was observed.⁹

However, mRNA often poorly correlates with protein expression either due to its degradation or inefficient translation.¹¹ The proteome also differs from cell to cell and constantly changes through its biochemical interactions in response to stimuli. Therefore, proteomic analysis of early changes in cisplatin-induced ototoxicity is key to understanding the corresponding functional state of the cell, as well as the mechanisms associated with hearing loss. The simultaneous analysis of multiple proteins involved in cellular survival or apoptosis will identify the pathogenic cellular pathways responsible for the evolution of ototoxicity associated with cisplatin treatment.

This study is the first to report the use of antibody microarrays, a powerful new tool, for simultaneously studying a multitude of cochlear proteins that change expression after cisplatin treatment. Antibody microarrays directly assay protein expressions and have been validated as a reliable tool for identifying and analyzing protein profiles in biological systems.^{12,13} The microarray used for our studies was spotted with 725 antibodies against proteins from a broad spectrum of signaling pathways. Changes in expression of 19 proteins, repeatable in two strains of rats, identified many novel ototoxic and cell survival responses before the observation of a significant functional or anatomical loss.

Experimental Design and Methods

Animals

Male Wistar and female Sprague—Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). The experimental protocol was reviewed and approved by the University at Buffalo Institutional Animal Care and Use Committee. The animals were housed and maintained in a temperature-controlled room with a 12-h light/dark cycle and allowed free access to food and water.

Cisplatin Administration

Cisplatin (Sigma Aldrich Chemical Co., St. Louis, MO) was administered in a single dose of 12 mg/kg body weight by slow intraperitoneal infusion of 1 mg/ mL in sterile saline (0.9%) at 10 mL/hr. Control animals were infused with an equal volume of saline. All of the animals were hydrated with 5 mL of subcutaneous injection of saline twice a day until they were sacrificed 48 h after cisplatin administration.

Physiological Measurements

Auditory brainstem response (ABR) thresholds and distortion product otoacoustic emission (DPOAE) at 2f₁-f₂ were measured for each animal before and after cisplatin administration. The animals were anesthetized with isoflurane (4% induction, 1.5% maintenance with 1 L/min

O₂) for ABR and DPOAE measurements. Subcutaneous differential needle electrodes were placed at the vertex (noninverting), below the test ear (inverting), and below the contralateral ear (ground). The sound stimuli, 1 ms tone bursts (2.5, 5, 10, 20, or 40 kHz, 0.5 ms rise-fall times) or 25 μ s clicks, were generated using Tucker Davis Technologies (TDT, Alachua, FL) SigGen software and TDT hardware consisting of a real-time processor, programmable attenuator, electrostatic speaker driver, and electrostatic pressure field speaker. The stimuli were presented to the external auditory meatus and the sound intensity varied in 5 dB intervals from above to below threshold. Two hundred stimulus presentations, delivered at 21/s, were averaged using a TDT real-time processor controlled by BioSig software (TDT) to obtain the ABR. Hearing threshold was defined as the lowest intensity of stimulation that yielded a repeatable waveform with identifiable peaks in the ABR waveform.

DPOAE stimuli were elicited with two primary tones f_1 and f_2 at an f_2/f_1 ratio of 1.2. The level of f_1 and f_2 were set at $L_2 + 10$ dB. L_1 level was varied from 70 to 25 dB SPL in 5 dB increments. Two IHS-3738 high-frequency transducers (Intelligent Hearing System, Miami, FL, USA) were used to deliver f_1 and f_2 to the ear via separate flexible tubes connected to a probe inserted into the ear canal. Sound pressure levels were measured at the cubic difference frequency ($2f_1 - f_2$) using an ER10B+ probe microphone (Etymotics Research, Inc., Elk Grove Village, IL) and hardware and software from Smart Distortion Product Otoacoustic Emission System version 4.53 (IHS). The output of the microphone was sampled at 40 kHz over a period of 204 ms; the spectrum of each sweep was computed and averaged over 32 nonrejected sweeps. The noise floor was measured in a 24 Hz band surrounding $2f_1 - f_2$ and f_2 was varied from 1 to 16 kHz.

Cochleogram

Animals were anesthetized by CO₂ inspiration until there was no response to a toe pinch and decapitated, and the temporal bones were quickly removed. The round and oval windows were opened, and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2, 4 °C) was slowly perfused through the round window for approximately one minute; then the cochlea was immersed in fixative for 3 h. Afterward, the specimens were stained with Harris' hematoxylin solution. The organ of Corti was dissected and mounted as a flat surface preparation in glycerin on a glass slide. Specimens were examined under a light microscope (400 $\times$) and the number of missing inner hair cells (IHC) and OHC were counted over 0.24 mm intervals along the length of the cochlea. Cochleograms showing the percent hair cell loss as a function of percent distance from the apex were constructed for each animal.¹⁴

Antibody Microarray

Cochlear protein samples from control and cisplatin injected rats were prepared 48 h after cisplatin injection so that the protein expression in the cochlea would reflect an early evolving stage of cisplatin-induced ototoxicity. Rats were anesthetized by CO₂ inspiration, and decapitated. Whole cochlea samples dissected from 4 cochleae, including the bony shell, lateral wall, basilar membrane, and modiolus, were homogenized with 150 μ L lysis buffer supplemented with protease and phosphatase inhibitors, each provided with the "Panorama Antibody Microarray - XPRESS Profiler 725 kit" (Sigma-Aldrich Corporation, St. Louis, MO). The protein concentration of the sample was determined using the BioRad (Bio-Rad, Hercules, CA) Bradford assay.¹⁵ Fluorescent dyes (Cy3 and Cy5 - GE Healthcare, Buckinghamshire, UK) were prepared as 800 μ M stock solutions in 0.1 M carbonate/bicarbonate buffer, pH 9.3, at room temperature. Cochlear proteins from the samples were labeled using an *N*-hydroxysuccinimide ester linkage to lysines by adding 7.5 μ L stock dye solution per 150 μ g protein adjusted to 1 mg/mL. Unlabeled dyes were removed by gel filtration using spin columns provided in the kit. Dye incorporation was determined spectrophotometrically at 552 nm (Cy3) and 650 nm (Cy5) using the molar extinction coefficient of 0.15 μ M⁻¹ cm⁻¹ at 552 nm for Cy3

and $0.25 \mu\text{M}^{-1} \text{cm}^{-1}$ at 650 nm for Cy5. To optimize the signal-to-noise ratio, labeling was repeated as necessary to achieve a molar dye/protein ratio >2 . Dye/protein molar ratio was calculated assuming an average molecular weight of 60 kDa. To control for differences in labeling stoichiometry, a dye-swapping paradigm¹⁶ was used. For each experiment, two slides spotted with antibodies were incubated with labeled protein samples for 0.5 h each at room temperature. The first slide was incubated with Cy3-labeled control sample and Cy5-labeled cisplatin-treated sample. A second identical slide was incubated with the opposite labeling scheme, that is, Cy5-labeled control sample and Cy3-labeled cisplatin-treated sample. Fluorescent signal intensities from the binding of Cy3- or Cy5-labeled protein were then recorded for each antibody spot using a GenePix Professional 4200A Microarray Scanner (Molecular Devices Corporation, Sunnyvale, CA). All antibody spots that showed a negative signal-to-noise ratio were deleted from further analysis. The data obtained was normalized by calculating the geometric mean of 4 background-corrected fluorescence intensities from duplicate antibody spots on each slide. The ratio of fluorescent intensity of cisplatin-treated samples to that of control samples (fold change induced by cisplatin) was calculated using the normalized fluorescent intensities.

Western Blot

Changes in protein level were quantified using Western blotting methods.¹⁷ Proteins were separated on 4–12% gradient NuPage gels (Invitrogen, Carlsbad, CA), transferred to polyvinylidene difluoride membranes, blocked with 0.1% I-Block (Applied Biosystems, Foster City, CA), and probed with antibodies of interest using chemiluminescence detection (Pierce Chemical Co., Rockford, IL). A Fuji model LAS 1000 imaging system (Stamford, CT) was used to visualize gel bands. Background corrected bands (NIH Image J software) were normalized against bands obtained by stripping the membrane with 25 mM glycine (BioRad), pH 2.0, 1% lauryl sulfate and reprobing with an antibody against actin. Actin was used as a housekeeping protein because cisplatin induced a change in the level of the widely used housekeeping enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunocytochemistry

Localization of nitrotyrosine in the cochlea, 48 h after cisplatin treatment was done by immunocytochemistry using confocal microscopy.¹⁷ Cochlear tissue was fixed in 10% buffered formalin for 1 h, and the organ of Corti was permeabilised in PBS + 1% (v/v) Triton X-100 for 30 min. Then the tissue was placed in blocking solution (5% v/v goat serum, 2% w/v BSA in PBS) for 1 h and incubated overnight at 4 °C in primary antibody (1:400), followed by incubation with the secondary antibody at room temperature for 1 h. The tissue was then colabeled with phalloidin that labels f-actin and TOPRO-3 that stains nuclei. The stained specimens were mounted on slides with ProLong Gold antifade reagent (P36934, Invitrogen Molecular Probes) and examined using the Carl Zeiss Laser Scanning Systems LSM 510. Images were captured and analyzed with Zeiss LSM Image Examiner (version 4,0,0,91, Carl Zeiss GmbH Jena). The cellular and subcellular distribution was assessed by using 3D analysis of the organ of Corti. Secondary antibodies conjugated to Alexa Fluor 488 were obtained from Invitrogen - Molecular Probes (Carlsbad, CA).

Results

Measures of Early Stage Ototoxicity

A goal of proteomics is to identify protein markers at an early stage in pathogenesis as potential drug targets for therapeutic intervention. A 12 mg/kg dose of cisplatin for rats will lead to substantial hearing loss within a weeks time.¹⁰ At the 48 h time point, physiological and morphological measures indicated that pathogenesis was still at an early stage.

ABR for stimuli at 2.5, 5, 10, 20, 40 kHz and for clicks revealed threshold shifts ranging from 10 to 34 dB for Wistar rats and 4 to 12 dB for Sprague—Dawley rats (Figure 1). In both strains, the level of cisplatin-induced hearing loss was greater at the higher (40 kHz) and lower (2.5 kHz) frequencies, whereas a lesser degree of functional loss was sustained in the mid frequencies (10, 20 kHz). These changes are likely to be irreversible, as similar studies with lesser dosage of cisplatin (5 mg/kg) have shown significant shift in hearing threshold at 3, 7, 30, and 90 days after cisplatin treatment.¹⁸ DPOAE amplitude decreased approximately 10 dB at 8 and 16 kHz (Figure 2) with no change at lower frequencies (not shown).

Cochleograms indicated minimal or no hair cell loss in regions sensitive to mid frequencies in both Wistar and Sprague—Dawley rats and minimal loss of outer hair cells at the base and apex (Figure 3). The small sizes of the lesions reflect a very early stage of cochlea pathology.

Protein Expression Profile

Analysis by antibody microarrays resulted in the detection of 581 proteins in Wistar rats and 626 proteins in Sprague—Dawley rats. Figure 4 shows the antibody array #1 labeled with Cy3 for the control tissues and Cy5 for the cisplatin treated tissues (left panel). The dye-swapped array #2 was labeled with Cy5 for the control tissues and Cy3 for the cisplatin treated tissues (right panel). The insets in the center panel show higher resolution images of respective segments of each array. Dye swapping minimizes bias due to labeling with two dyes. The pseudocolor images depict the ratio of fluorescence intensities of protein in control vs experimental samples.

Approximately 80% of the 725 antibodies on the arrays were detected for both strains of rats; 302/309 proteins exhibited a ≥ 1.1 fold increase, 214/261 exhibited a ≥ 0.9 fold decrease, and 65/56 remained unaltered (fold change was 1.0, e.g., actin) in Wistar/Sprague—Dawley rats (Figure 5). Levels of 15 proteins increased in both strains by ≥ 1.5 fold, whereas 4 proteins decreased by ≥ 0.6 fold (Table 1). Cisplatin-induced changes in 10 proteins were associated with a survival response (increased expression of ATF2, JAB1, Mdm2, Rsk1, SUMO-1, myosin VI, p21WAF1Cip1, PRMT4, and reelin and decreased expression of active caspase 3). Increased expression of 4 proteins (Tal, Granzyme B, SLIPR/MAGI3, RIP) and decreased expression of 3 proteins (EGF - epidermal growth factor, p35, ubiquitin C-terminal hydrolase L1) were linked to cell death responses. However, it is unclear if two cytoskeletal proteins, centrin and neurofilament 68, are associated with either survival or apoptosis responses.

Validation

Immunoblot analysis of cisplatin-treated rats confirmed the increased expression of ATF2, RIP, and nitrotyrosine (Figure 6). In contrast, actin levels remained constant in agreement with microarray results (1.0 ± 0.2 fold change for combined Wistar and Sprague—Dawley data). We used actin as a normalizing protein, rather than GAPDH, a typical housekeeping enzyme, because cisplatin induced a change in GAPDH levels in both microarray and immunoblot experiments. Surprisingly, the anti-nitrotyrosine immunoblot was dominated by only two protein bands at 70 and 75 kDa.

Immunocytochemistry studies were carried out to localize the expression of nitrotyrosine in the sensory epithelium. Immunofluorescent labeling of nitrotyrosine was present in some, but not all, stereocilia (S), in Hensen's cells (H), in outer pillar cell nuclei (P), as well as in tunnel crossing fibers (Figure 7F). Low-level labeling was observed in some, but not all, Deiters' cell and inner pillar cell nuclei. Stereocilia labeling was not due to nonspecific binding of secondary antibody because it was not observed in control specimen labeled with an unrelated primary antibody (Figure 7D). Nitrotyrosine labeling in Hensen's cells was focal and appeared to be in the Golgicentrosome perinuclear region (Figure 7, panel A and B, red perinuclear foci).

Discussion

This study is the first to use a high-throughput antibody microarray to identify changes in protein expression levels in the inner ear. The proteomic analysis of cisplatin ototoxicity revealed the involvement of several novel proteins not previously reported in studies of either normal or pathological hearing. Cisplatin-induced strong expression changes in 19 proteins that either increased by ≥ 1.5 or decreased by ≥ 0.6 fold in both Wistar and Sprague—Dawley rats (Table 1). Only 4 of these proteins have been reported in previous studies in the cochlea, myosin VI,¹⁹ neurofilament 68,²⁰ caspase 3,²¹ and epidermal growth factor.²² Fifteen proteins have heretofore never been identified in cisplatin-induced ototoxicity. Review of the literature indicates that the cisplatin-induced changes in expression of 10 proteins are associated with a survival response, 7 indicate a cell death response, and 2 proteins have an undetermined role.

Of the 10 proteins that reflect a survival response, 5 are related to the p53 signaling pathway. p21, myosin VI, and Mdm2 are transcriptionally regulated by p53. p21 is a member of the cip/kip family of cyclin kinase inhibitors that function in cell cycle arrest. It acts as an anti-apoptotic and growth-promoting protein.²³ Myosin VI also functions in p53 mediated cell survival associated with Golgi integrity.²⁴ It is a minus-end-directed motor, abundant in hair cells and essential for development and maintenance of stereocilia.¹⁹ The Mdm2 (murine double minute) oncogene represses p53 transcriptional activity by binding to and blocking the N-terminal trans-activation domain of p53. It also acts as an E3 ubiquitin ligase targeting p53 by poly ubiquitination for exit from the nucleus and degradation by the 26S proteasome.²⁵ Additionally, Mdm2 can initiate the intrinsic apoptotic pathway, because monoubiquitination signals transport p53 to the mitochondria.²⁶ Like Mdm2, JAB1 (jun activation domain-binding protein 1) also functions as a nuclear export mediator in the degradation of p53.²⁷ SUMOs (small ubiquitin-related modifiers) are reversible post-translational protein modifiers that change the localization, activity, and stability of the protein to which they are covalently bound.²⁸ Increased expression of SUMO-1 48 h following cisplatin administration is consistent with an anti-apoptotic sumoylation that enhances Mdm2's ability to ubiquitinate p53.

Cisplatin-induced changes in 5 other proteins, not apparently linked to p53, also indicate a survival response. Expression levels increased for 4 survival-associated proteins (ATF2, Rsk1, PRMT4, and reelin). ATF2 is a member of the ATF/cAMP response element-binding (CREB) protein family. DNA damage is one of the classic inducers of ATF2 transcriptional activity.²⁹ ATF2 is implicated in the activation of a large set of genes important in drug resistance and in the regulation of ER stress regulatory protein Grp78.³⁰ Rsk1 is a member of the p90 ribosomal S6 kinase (Rsk) family which activates ATF/CREB family transcription factors and the transcriptional coactivator CREB-binding protein.³¹ PRMT4 (Protein arginine N-methyltransferase-4) is a promoter-specific regulator of NF- κ B recruitment to chromatin in cell survival responses and also plays a key role in RNA transport and splicing.³² Reelin is an extracellular matrix protein that activates a survival response through p35/cyclin-dependent kinase 5 (Cdk5) and inactivates an apoptotic response through the Src-tyrosine kinase family member Fyn.³³ Expression levels decreased for active caspase 3, a major effector in neuronal apoptosis triggered by various stimuli,³⁴ whereas Bcl-2 remained unaltered. However, a marked increment in active caspase 3 accompanied by a decrement in Bcl-2 has been reported with a lesser dosage (5 mg/kg) of cisplatin.³ It is not clear whether the dose difference causes this discrepancy, even though an *in vitro* study that compared the effects of a higher and lower dosage of cisplatin, reported a decline in caspase 3 activity with the higher dosage.³⁵ This unexpected complexity must be resolved in future experiments, as the time point chosen for the study may also have a critical role in determining the delicate balance between survival or apoptotic responses.

In contrast to changes promoting cell survival, increases observed in granzyme B, Tal, SLIPR/MAGI3, and RIP suggest an apoptotic response. Granzymes are structurally related serine proteases. Granzyme B is responsible for the rapid activation of pro-apoptotic protein Bid. It can induce cytochrome c release by cleavage and inactivation of the anti-apoptotic Bcl-2 family member Mcl-1.³⁶ Apoptosis from granzyme B mediated cytochrome c release can also proceed in a caspase-independent manner.³⁷ It is interesting to speculate that such a response might be related to the cisplatin-induced decrease in active caspase 3. Tal (Tsg101-associated ligase) polyubiquitinates the tumor susceptibility gene 101 (Tsg101) product resulting in proteasomal degradation. Tsg 101 is essential for cell survival,³⁸ endosomal sorting, membrane receptor degradation, and the final stages of cytokinesis.³⁹ SLIPR/MAGI3 is a membrane-associated guanylate kinase protein, which localizes transmembrane proteins to specific sites. SLIPR/MAGI3 interacts with protein phosphatase PTEN, a tumor suppressor, to antagonize the survival activity of protein kinase B/Akt.⁴⁰ RIP (receptor interacting protein) is a 74 kDa Ser/Thr kinase. It interacts with other regulatory proteins in a signaling scaffold associated with the tumor necrosis factor receptor, which is capable of signaling either the induction of apoptosis, through the activation of caspase 8, or the anti-apoptotic NF- κ B pathway. It is a cell death domain adapter protein that can bind to the adapter proteins TRADD, RAID (CRADD), and TRAF2.⁴¹

The decrease in the expression of EGF, p35, and Ubiquitin C-terminal hydrolase L1 also indicates a cisplatin-induced cellular death response. EGF receptor activity can activate the extracellular signal-regulated kinase/mitogen-activated protein kinase cascade, which functions in organogenesis and in tissue homeostasis.⁴² p35 is an activator of Cdk5, which displays kinase activity in postmitotic neurons. Dysregulation of Cdk5 has been implicated in neurodegeneration.⁴³ The expression of p35 is induced in differentiated neurons and is enhanced by extracellular stimuli such as neurotrophic factors or extra-cellular matrix molecules.⁴⁴ A decrease of p35 concomitant with increased levels of reelin would not be expected³³ and indicate a more complex inter-relationship than might have been predicted. Ubiquitin C-terminal hydrolase L1 is a member of the ubiquitin carboxy-terminal hydrolase family of deubiquitinating enzymes. It is a multifunctional protein in neurons that can hydrolyze bonds between ubiquitin and substrate proteins, thereby reversing the functional state of the substrate.⁴⁵

Cytoskeletal proteins centrin and neurofilament 68 also increased, but their role in cisplatin ototoxicity is unclear. Centrans are members of the EF-hand family of Ca²⁺-binding proteins.⁴⁶ It has been suggested that Cen3 participates in centrosome reproduction and duplication, whereas Cen1/Cen2 play a role in centriole separation preceding centrosome duplication during the cell cycle.⁴⁷ Cen2 also stimulates nucleotide excision repair and might therefore promote survival.⁴⁸ Neurofilament 68 is one of the five major subunits of intermediate filaments expressed in neurons. Cisplatin upregulates the expression of NEFL encoding this 68 kDa neurofilament protein.⁴⁹ During axonal growth, neurofilament subunits are incorporated all along the axon in a dynamic process. Imbalances in subunit stoichiometry have been implicated in the induction of neurodegeneration characterized by neurofilamentous aggregates.⁵⁰

Nitrotyrosine expression indicates cisplatin-induced post-translational oxidative modification. Cisplatin-induced expression of nitrotyrosine (1.4–2.1 fold change) was observed by antibody microarray and confirmed by immunoblotting. Even though nitrotyrosine did not meet the criterion of >1.5 fold increase for both strains, investigation of its expression was carried to the level of subcellular localization before other proteins because nitration of tyrosine residues resulting from the activity of either peroxynitrite or nitrogen dioxide is known to produce catastrophic effects on protein function. For example, nitration of tyrosine blocks the ability of protein tyrosine kinases to activate certain transcription factors.⁵¹ Several nitrated proteins have been reported in other systems,⁵² but none have been reported in the cochlea so far. The

strong expression of the 70/75 kDa protein(s) found in immunoblotting, as well as localization of nitrotyrosine in the stereocilia of OHC, supporting cells, and tunnel crossing fibers, reflect, to our knowledge, the first report of evidence of stress-induced oxidative damage to cochlear proteins.

Conclusion

Identification of several novel proteins involved in cell death or survival responses during this early stage of ototoxicity indicates that multiplex microarrays are a sensitive and revealing tool for understanding the molecular pathogenesis of cisplatin-induced hearing loss. These results illuminate the delicate balance between early survival and apoptotic responses in cisplatin ototoxicity that take place before significant functional changes occur. Therapeutic interventions that target proteins associated with the onset of ototoxicity, such as those identified here, are likely to be far more effective than those that attempt to block the later stages of cell death.

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Figure 1.

Hearing threshold after cisplatin treatment. The ABR recorded before and 48 h after cisplatin treatment shows threshold shifts ranging from 10–34 dB in (A) Wistar and 4–12 dB in (B) Sprague—Dawley rats at 2.5, 5, 10, 20, 40 kHz and clicks. Measurements were done for $n = 6$ rats for the pretreatment group and $n = 3$ for the cisplatin treated group. Results are expressed as mean $\pm$ standard deviation (SD).

Figure 2.

Cisplatin-induced DPOAE changes in Wistar rats. DPOAEs showed a decrease of approximately 10 dB at 8 and 16 kHz in both (A) Wistar and (B) Sprague—Dawley rats 48 h after cisplatin treatment. The pre-NF and post-cis-NF traces indicate the noise floor in DPOAE recordings before and after treatment with cisplatin. The measurements were done in $n = 6$ rats for the pretreatment group and $n = 3$ for the cisplatin treated group. The results are expressed as mean $\pm$ SD.

Figure 3.

Hair cell loss 48 h after cisplatin treatment. Cochleograms indicated that 12 mg/kg cisplatin induced minimal or no hair cell loss at 48 h post-treatment in both (A) Wistar and (B) Sprague-Dawley rats. The cochleograms from the right ear are given though the left ears also indicated a similar pattern (data not shown).

Figure 4.

Antibody microarray of cisplatin-induced changes in cochlea. For array # 1, control proteins from Sprague—Dawley rats were labeled with Cy3 and proteins from cisplatin-treated rats with Cy5. For array # 2, the labeling dyes were swapped (controls labeled with Cy5 and cisplatin-treated with Cy3). Green spots indicate Cy5-labeled proteins outnumber Cy3-labeled proteins. Red spots indicate Cy-3 labeled proteins dominate. Similar results were obtained for samples from Wistar rats (figure not shown).

Figure 5. Cochlear protein expression profile after cisplatin treatment. (A) Wistar and (B) Sprague—Dawley: plots of number of proteins (ordinate) versus fold changes in protein expression. Values greater than 1 indicate an increase in protein level in cisplatin treated group versus control. Values less than 1 reflect a decrease in protein expression. The pattern of distribution in terms of the number of proteins that increased or decreased after cisplatin treatment was similar in both strains. Cut-off lines at fold changes of 0.6 and 1.5 indicate the selection criteria for further protein analysis.

Figure 6.

Validation of protein expression by immunoblotting; 60 μ g of protein from Sprague—Dawley cochleae were loaded in each lane for control or cisplatin-treated rats. Immunoblotting results indicate cisplatin-dependent increases in the expression of (A) ATF2, (B) RIP, and (C) nitrotyrosine, whereas (D) shows the expression of actin. Numbers give apparent molecular weight in kDa.

Figure 7.

Cisplatin-induced localization of nitrotyrosine in cochlea. Images, obtained using confocal microscopy, indicate the presence of nitrotyrosine in stereocilia (*S*), Hensen's cells (*H*), outer pillar cell nuclei (*P*), and tunnel crossing fibers (*F*). Sprague—Dawley sensory epithelium was labeled with anti-nitrotyrosine, 48 h after cisplatin treatment. Red staining indicates nitrotyrosine, green indicates f-actin, and blue indicates nuclei. The confocal sections are at the level of (A) the stereocilia, (B) outer hair cell nuclei, and (C) Deiters' and pillar cell nuclei. The negative control (D), labeled with a different primary, rules out nonspecific labeling of the stereocilia by the secondary antibody.

Table 1

Proteins with Major Changes after Cisplatin Treatment

protein	function			antibody microarray fold change		
	apoptosis	survival	cell cycle/cytoskeleton	Wistar	SD ^a	mean
ATF2		Y		1.6	1.5	1.6
IAB1		Y		1.6	1.6	1.6
Tal	Y			1.5	1.7	1.6
Mdm2		Y		1.7	1.5	1.6
Granzyme B	Y			1.8	1.6	1.7
Rsk1		Y		1.7	1.7	1.7
Myosin VI		Y		1.5	1.9	1.7
SUMO-1		Y		1.9	1.5	1.7
p21WAF1Cip1		Y	Y	1.6	1.9	1.7
PRMT4		Y		1.8	1.7	1.7
SLIPR MAGI3	Y			1.5	2.1	1.8
Reelin		Y		2.1	1.6	1.8
Receptor Interacting Protein	Y	Y		2	2	2.0
Centrin			Y	1.8	2.4	2.1
Neurofilament 68			Y	1.8	2.4	2.1
Caspase 3 Active	Y			0.6	0.3	0.4
Epidermal Growth Factor		Y		0.5	0.5	0.5
p35 Cdk5 Regulator		Y		0.3	0.6	0.5
Ubiquitin C terminal Hydrolase L1		Y		0.6	0.6	0.6
Nitrotyrosine ^b	Y			1.4	2.1	1.8

^a Cisplatin induced changes in 19 proteins that increased by ≥ 1.5 fold or decreased by ≥ 0.6 fold in both Wistar and Sprague-Dawley (SD).

^b Even though nitrotyrosine did not meet the selection criteria for the table, it was included due to its functional significance in cisplatin-induced ototoxicity.